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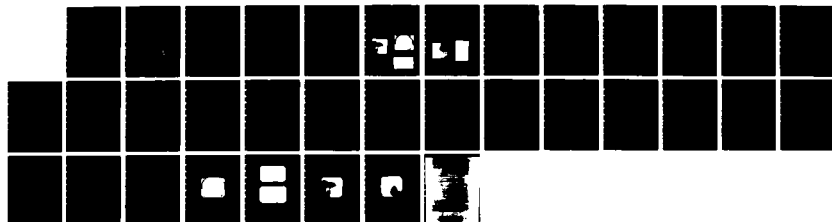
BIOLOGIC ACTIVITY OF TYPE I AND TYPE II FUSOBACTERIUM  
NUCLEATUM ISOLATES F. (U) ARMY INST OF DENTAL RESEARCH  
WASHINGTON DC J W VINCENT ET AL. 26 JUL 84

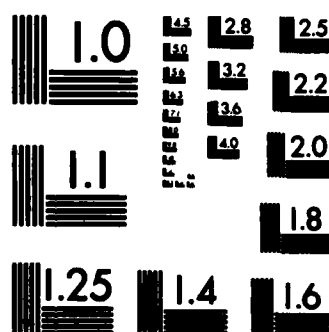
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Biologic Activity of Type I and Type II

Fusobacterium nucleatum Isolates

From Clinically Characterized Sites.

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1. Request clearance for publication of the attached manuscript entitled "Biologic Activity of Type I and Type II Fusobacterium nucleatum Isolates From Clinically Characterized Sites."
2. If approved for publication, the journal of choice is the Journal of Periodontology.

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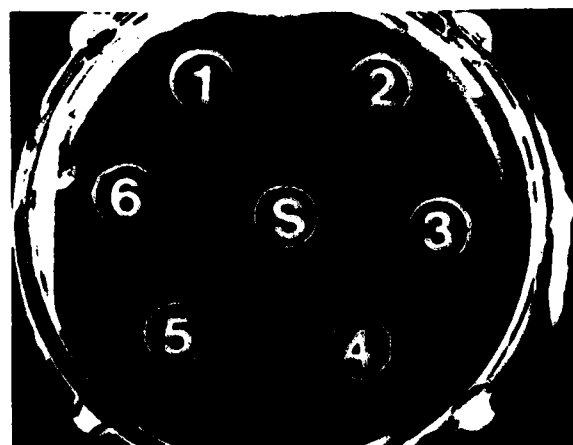
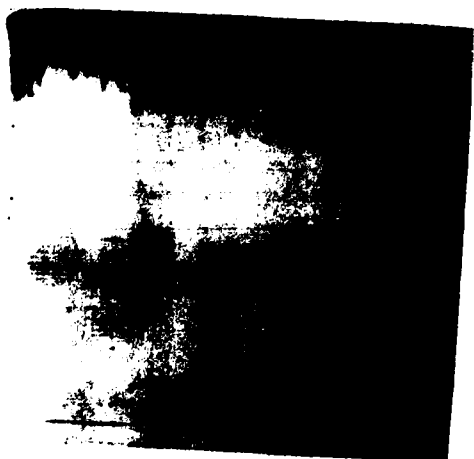
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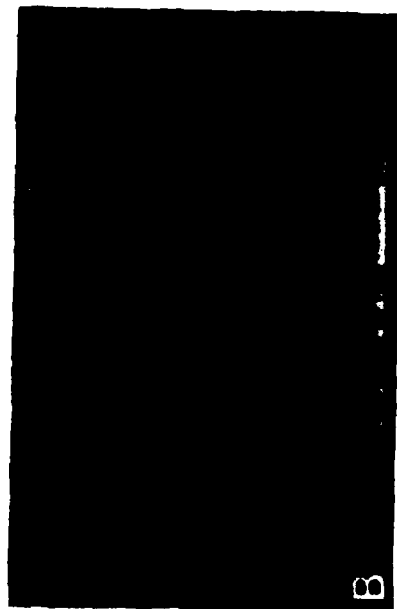
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Biologic Activity of Type I and Type II

Fusobacterium nucleatum Isolates

From Clinically Characterized Sites

## ABSTRACT

Fusobacterium nucleatum is a microorganism commonly cultured from periodontal disease sites. F. nucleatum isolates (120) were obtained from subgingival plaque samples taken from 27 clinically characterized sites utilizing a selective culture medium. All isolates were verified by morphology, Gram-stain reactions, oxygen tolerance, and biochemical reactions. A total of eight clinical isolates and two typed strains were utilized for further evaluation. In this study, there was no relationship found between GI and probing depth or between probing depth and frequency of isolation of Type I or Type II F. nucleatum colonies. There was a significant increase in isolation of Type II colonies with a GI of 2 ( $P < .0001$ ). All isolates tested shared lines of identity by double diffusion in agar and displayed similar ability to hemagglutinate sheep erythrocytes and a reduction in this hemagglutination activity by previous exposure to 50 mM D-galactose. All isolates tested showed similar protein patterns as determined by polyacrylamide gel electrophoresis. By the methods utilized, no differences were detected between Type I and Type II F. nucleatum; however, there is a statistically significant increase in Type II isolates with increasing levels of gingivitis.

Fusobacterium nucleatum is a Gram-negative anaerobic rod isolated frequently from human dental plaque. Numerous cultural studies have identified the presence of this organism in increased numbers in certain human periodontal diseases. It has been isolated from early<sup>1</sup> and advanced periodontitis,<sup>2-3</sup> juvenile periodontitis,<sup>4</sup> and chronic gingivitis.<sup>5</sup>

The significance of F. nucleatum in these various disease states has been evaluated in numerous serological studies. Evans et al.<sup>6</sup> demonstrated the presence of a bacteriocidal antibody in mammalian serum reactive with isolates of F. nucleatum. Kristoffersen<sup>7</sup> described a group reactive antigen which was reactive with antibodies in human sera. Hofstad<sup>8</sup> identified antibodies reactive with the lipopolysaccharide of several Gram-negative organisms including F. nucleatum. These antibodies were isolated from the serum of normal human patients. More recently Falkler et al.<sup>9</sup> demonstrated humoral IgG, IgA, and IgM responses to three typed strains of F. nucleatum. The results indicated that higher levels of IgG and IgA antibody reactive to all three typed strains were observed in chronic periodontitis patients while the level of IgM antibody showed no differences between chronic periodontitis patients and healthy controls. In a study evaluating strains of F. nucleatum isolated from various human disease states and from healthy controls, it was observed that all clinical isolates shared lines of identity when tested

with positive serum by double diffusion in agar.<sup>10</sup> These human isolates of F. nucleatum, however, did demonstrate antigenic differences from isolates identified culturally and biochemically as F. nucleatum obtained from other animal species. These results suggest that isolates of F. nucleatum from various disease states in humans share antigenic determinants that differ from F. nucleatum strains isolated from different animal species.

A recent study of DNA-DNA hybridization of 16 strains of F. nucleatum and five other strains representing other Fusobacterium species concluded that F. nucleatum comprised a heterogeneous group of microorganisms closely related to Fusobacterium periodontium and Fusobacterium simai but unrelated to the other species of Fusobacterium which were tested by this technique.<sup>11</sup>

The interactions of F. nucleatum with various components of the oral environment have been discussed. F. nucleatum has been shown to demonstrate a characteristic hemagglutinating activity (HA) which can be inhibited by specific antiserum.<sup>12</sup> This HA can be reduced by the activity of D-galactose,<sup>13</sup> human salivary mucinous glycoproteins,<sup>14</sup> and adsorption with Streptococcus and Bacteroides.<sup>15</sup> F. nucleatum cell fragments attach to oral epithelial cells, gingival fibroblasts, and white blood cells;<sup>16</sup> serum non-antibody glycoproteins attach to F. nucleatum found in periodontal diseases.<sup>17</sup> These characteristics suggest possible mechanisms by which F. nucleatum is able to colonize the oral

environment and thereby serve as an antigenic stimulus via the humoral arm of the immune system. The ability of this micro-organism to activate the alternate complement pathway<sup>18</sup> provides an additional mechanism whereby a role in the immunopathology of periodontal diseases can be identified.

A culture medium, crystal violet-erythromycin agar (CVE) has been described for selective isolation of F. nucleatum from dental plaque.<sup>19</sup> Two colonial morphologies were described as: Type I, a 2 mm transparent, smooth, blue colony having an entire edge with a dark blue center; and Type II, a 1 to 2 mm transparent round or irregular blue colony with a speckled appearance.

The purpose of this study was to determine the frequency of occurrence of F. nucleatum isolates of these two colonial morphologies in various human disease states and to compare these isolates culturally, biochemically and antigenically. Protein profiles were also determined by polyacrylamide gel electrophoresis (PAGE).

#### MATERIALS AND METHODS

Patient population. Plaque samples were harvested from a total of 27 sites in eight patients. All sites were characterized utilizing Gingival Index<sup>20</sup> and probing depth measurements. Patients were screened to eliminate systemic complicating factors or antibiotic therapy within the previous six months.

Cultures and cultural conditions. Prior to sampling, all

supragingival plaque was removed and a sterile Gracey curette\* was used to obtain subgingival plaque from the base of the gingival sulcus/pocket. The plaque sample was immediately placed in 2 ml of pre-reduced, sterile reduced transport fluid<sup>21</sup> (RTF) containing 0.05% cysteine-HCl (H<sub>2</sub>O) in place of dithiothreitol. After agitation by vortexing for 30 seconds, serial tenfold dilutions in RTF were made and a 100 µl sample was immediately plated on pre-reduced CVE<sup>19</sup> agar containing (per liter): Trypticase, 10.0 g; yeast extract, 5.0 g; sodium chloride, 5.0 g; glucose, 2.0 g; tryptophan, 0.2 g; agar, 15.0 g; crystal violet, 0.005 g; erythromycin, 0.004 g; and defibrinated sheep blood, 50 ml at a final pH of 7.2. All samples were incubated anaerobically for four days at 37°C utilizing the GasPak system.\*\* After incubation, isolates were selected by colonial morphology and tested for verification of species. All isolates were streaked on two blood agar plates with one sample incubated aerobically and one sample incubated anaerobically for 48 hours at 37°C. Biochemical reactions were determined with the API 20A system.\*\*\* Each isolate was observed by phase-contrast microscopy and Gram stained to verify the typical morphology. The

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\*Hu Friedy, Chicago, IL

\*\*BBL Microbiology Systems, Cockeysville, MD

\*\*\*Analytab Products, Plainsville, NY

only isolates utilized were those which demonstrated Gram-negative staining of long, pointed rods, failure to grow aerobically on blood agar and giving the characteristic biochemical results on the API 20A system (positive indole response, all other tests negative).

Eight clinical isolates of F. nucleatum (four with Type I and four with Type II colonial morphology) and two typed strains (ATCC 10953 and VPI 4355) were utilized for further characterization. All cultures were grown anaerobically at 37°C in a pre-reduced modified tryptone medium<sup>12</sup> containing (per 100 ml) tryptone, 10.0 g; yeast extract, 5.0 g; glucose, 0.2 g; thioglycolate, 0.5 g; and MgSO<sub>4</sub>, 0.125 g. Cells were harvested by centrifugation at 10,000 X g for 10 minutes at 4°C, washed three times in 0.01 M phosphate-buffered saline containing 0.15 M NaCl (PBS) (pH 7.2), resuspended at a concentration of 600 Klett units utilizing the Klett-Summerson Photoelectric Colorimeter<sup>+</sup> with a red filter, and then were designated as the whole cell preparations (WC). Each WC was sonicated using the Heat Systems Sonicator<sup>++</sup> using the microtip setting, 8 bursts of 30 seconds each, and these were designated as sonicated preparations (SP).

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<sup>+</sup> Klett Mfg. Co, Inc., New York, NY

<sup>++</sup> Heat System Ultrasonics, Inc., Plainview, NY

Immunodiffusion. SP of all isolates and the two typed strains of F. nucleatum were reacted by double diffusion in 1% agarose<sup>22</sup> in 0.01 M PBS (pH 7.2) with an undiluted rabbit anti-Fusobacterium nucleatum 10953 serum.<sup>12</sup> Reactions were incubated for 24 hours at 4°C in a humidior, then observed for visible bands of precipitation.

Hemagglutination. The WC (50 µl) were placed in the first well of microtitration multiwell plates<sup>+++</sup> and serial twofold dilutions were performed in 25 µl of PBS after which the volume in each well was restored to 50 µl by the addition of PBS. Next, 25 µl of a 1.25% suspension of freshly washed sheep erythrocytes was added to each well; the plates were mixed for 20 seconds, and then incubated for 30 minutes at 37°C followed by 1 hour at 4°C. Titers were determined as the reciprocal of the highest dilution demonstrating a 2+ HA. HA inhibition was determined as described above except for the addition of 50 mM D-galactose (25 µl) in place of PBS and incubated for 30 minutes at 37°C prior to adding the 25 µl of 1.25% sheep erythrocytes.

Polyacrylamide gel electrophoresis (PAGE). A modification of the method of Moore et al.<sup>23</sup> was utilized to evaluate soluble proteins. Briefly, 2 ml of each WC was pelleted by centrifugation

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<sup>+++</sup> Limbo Scientific, Inc., Hamden, CT



at 10,000 X g for 10 minutes at 4°C and then restored to 2 ml in electrophoresis buffer containing (per liter) Tris, 10.75 g; boric acid, 5.0 g and Na<sub>2</sub> EDTA, 0.93 g (pH 8.4), and subjected to sonication as previously described. Each sample was heated to 55°C for 5 minutes in a water bath and then subjected to centrifugation at 8,000 X g for 10 minutes. The resulting supernatant fluid was prepared for electrophoresis by adding one third of its volume of sucrose and 0.5 ml of 1% bromophenol blue as a tracker dye. Samples, 25 µl, were applied to wells in a PAA 4/30 gradient gel<sup>§</sup> previously equilibrated in the electrophoresis buffer by running for 20 minutes at 70 volts utilizing a Pharmacia Gel Electrophoresis apparatus GE-4 ll with an EPS 500/400 power supply.<sup>§</sup> The samples were electrophoresed at 500 volts for 15 minutes to sharpen the sample band, then at 125 volts until the tracker dye approached the bottom of the gel (approximately 2 1/2 hours). This technique was utilized to evaluate the low molecular weight proteins (<50,000 Daltons). Larger proteins (50,000—1,000,000 Daltons) were evaluated as above, except that electrophoresis conditions were initially 70 volts for 15 minutes followed by 150 volts for 16 hours. The gels were removed and

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<sup>§</sup> Pharmacia Fine Chemicals, Piscataway, NJ

fixed in 10% sulfosalicylic acid for 30 minutes. Coomassie Blue R-250 was used as a staining solution containing 0.1% Coomassie Blue R-250 in 25% absolute methanol, 10% acetic acid, and 65% distilled water (V-V-V) and filtered immediately before use. The gels were stained at room temperature for 2 1/2 to 3 hours by diffusion. Destaining was accomplished by diffusion using 25% absolute methanol, 10% acetic acid and 65% distilled water (V-V-V). The protein patterns were determined using a Zeineh Soft Laser Scanning Densitometer<sup>§§</sup> and all slab gels were photographed.

## RESULTS

Frequency of isolation of Types I and II. (See Table 1.) F. nucleatum was isolated from 24 of 27 sites sampled. Of the 120 isolates, 49 demonstrated Type I colonial morphology, while 71 demonstrated Type II colonial morphology. Of 11 sites with a GI of 1 or less, Type I colonies comprised 26 isolates, while Type II colonies comprised 12 isolates. Of 16 sites with a GI of 2, Type I colonies comprised 23 isolates, while Type II colonies comprised 59 isolates (Table 2). Of the three sites which failed to yield F. nucleatum isolates, two presented with a GI of 1, while one site was judged to have a GI of 2. When sample sites

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<sup>§§</sup> Biomed Instruments, Inc., Chicago, IL

were grouped according to probing depths, it was observed that those sites with a probing depth of 2 mm or less (10) displayed 15 Type I isolates and 29 Type II isolates, while those sites with probing depths greater than 2 mm (17) displayed 34 Type I isolates and 42 Type II isolates (Table 3). Statistical analysis utilizing the Mantel-Haensel Chi-square was performed after sample sites were grouped by  $GI \leq 1$  or  $GI > 1$  and probing depths  $\leq 2$  mm or  $> 2$  mm. The relatedness of GI to probing depth was not statistically significant ( $P > .05$ ). When the frequency of isolation of Type I and II F. nucleatum was compared to probing depths, no significant differences were observed between groups with probing depths  $\leq 2$  mm and those with probing depths  $> 2$  mm ( $P > .05$ ) (Table 3). When the types of F. nucleatum isolates were compared to GI, there was a statistically significant shift from Type I to Type II isolates which accompanied an increase in GI from 1 to 2 ( $P < .0001$ ) (Table 2).

Immunodiffusion. All clinical isolates, regardless of colonial morphology, shared lines of identity with both typed strains of F. nucleatum when reacted with rabbit anti-F. nucleatum 10953 serum and with each of the other clinical isolates (Figure 1). These results suggest the presence of shared antigenic determinants among the clinical isolates and typed strains when evaluated by this technique.

Hemagglutination. All samples displayed 2+ hemagglutination

at a titer of 1:128 (Table 4) except one Type I isolate which displayed a titer of 1:256. All clinical samples previously incubated for 30 minutes at 37°C with 50 mM D-galactose displayed a titer of 1:32 (Table 4) while both typed strains showed a titer of 1:64. All samples possessed a similar ability to spontaneously hemagglutinate sheep erythrocytes (Figure 2) and a similar reduction in titer by prior exposure of the microorganisms to D-galactose.

Polyacrylamide gel electrophoresis. The electrophoretic pattern of soluble proteins of both low molecular weight (<50,000 Daltons) (Figure 3) and high molecular weight (>50,000 Daltons) (Figure 4) was similar in all isolates. As has been previously reported,<sup>23</sup> a problem of smearing of bands resulting in a high background staining was observed due to the presence of contaminating lipids. Some samples displayed bands slightly greater in intensity, but all bands in each sample showed identical migration patterns when subjected to electrophoresis in a gradient gel.

## DISCUSSION

By the techniques used in this study, all clinical isolates of F. nucleatum appeared to be identical regardless of the colonial morphology observed on CVE agar. Previous studies of DNA-DNA hybridization reported F. nucleatum isolates to be heterogeneous in nature.

The sharing of lines of identity by all clinical isolates and the two typed strains shows that the major antigenic determinants of these different isolates are similar. This suggests that the immune response elicited by these antigenic determinants would be similar. The intent of the immune response in this situation is protective in nature but could result in a hypersensitivity reaction that is in effect destructive and therefore contributory to the immunopathology of periodontal diseases. The ability of cell fragments of F. nucleatum to attach to various cell populations suggests a possible role in which an antibody population is stimulated and reacts with these bacterial fragments attached to host cells. The subsequent reaction may result in the activation of complement and the end result could be host cell lysis and chemotaxis of polymorphonuclear leukocytes. This scenario suggests one attractive explanation of the histological picture of the progression of periodontal diseases. The fact that all isolates displayed a similar ability to bind sheep erythrocytes utilizing D-galactose sensitive receptors demonstrates an equal ability of all isolates to attach to cell surface glycoproteins containing D-galactose. For an organism to colonize a given environment, it must be able to develop an attachment to this econiche; otherwise, it will simply be washed from the site. Although it was not evaluated in this study, it has been shown that F. nucleatum organisms can also attach to Streptococcus and Bacteroides

species. Members of these genera are commonly isolated from dental plaque. This characteristic could also play an important role in the sequential colonization of the gingival sulcus by microorganisms. Once present in the sulcus, it would appear from the immunodiffusion results that the major antigens of all clinical isolates and the two typed strains share identity and thus would be expected to elicit a similar immune response in the host whether protective or destructive in nature.

Finally, in our hands, PAGE of soluble proteins failed to detect differences between Type I and Type II F. nucleatum isolates. An explanation of the phenotypic difference of colonial morphology on CVE agar was not provided by any of the techniques utilized in this study. Type I and Type II F. nucleatum appeared identical. There was a statistically significant shift in the type of F. nucleatum isolated from sites with differing GI. The tendency to isolate Type II F. nucleatum more commonly from sites with a GI of 2 may demonstrate a shift in types of microorganisms which may differ in some characteristic other than those evaluated in this study. Such differences may be significant in the progression of periodontal diseases not readily detectable unless colonial morphology is considered in cultural evaluations.

#### MILITARY DISCLAIMER

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TABLE 1

Classification of *F. nucleatum* Isolates From  
Clinically Characterized Sites

Subjects	Sample Site Tooth/Surface*	Gingival Index	Probing Depth	Isolates Obtained (Type I) (Type II)	
36 yr. old male	2 D	0	5	0	0
	18 D	1	3	3	2
	19 D	2	3	1	6
	7 M	0	1	1	0
23 yr. old male	21 L	2	2	2	7
	19 D	2	3	0	6
	14 M	2	3	0	0
	15 D	2	2	1	8
33 yr. old male	31 M	2	2	3	4
	14 M	2	3	2	4
	1 M	2	6	4	4
	12 M	1	4	1	2
58 yr. old male	30 D	2	3	2	2
	23 L	2	2	3	1
	2 M	2	2	0	3
	11 B	1	2	0	0
41 yr. old male	3 L	2	2	0	2
	5 M	1	3	6	2
	31 M	2	3	1	6
	22 D	2	3	0	3
22 yr. old female	2 D	0	1	4	1
	22 M	1	2	1	3
33 yr. old male	31 D	2	4	0	1
	20 D	2	3	4	2
36 yr. old male	31 D	0	6	5	1
	18 D	0	5	4	1
	2 D	1	6	1	0
Total	8	27		49	71

\* M = mesial, D = distal, L = lingual and B = buccal

TABLE 2

Relatedness of GI to Colonial Morphology  
of F. nucleatum Isolates

Gingival Index	Colonial Morphology	
	Type I	Type II*
GI $\leq$ 1	26	12
GI > 1	23	59
Total	49	71

\* Statistically significant increase in frequency of isolation with GI > 1  
(P<.0001)

TABLE 3

Relatedness of Probing Depth to  
Colonial Morphology  
of F. nucleatum Isolates\*

Probing Depth (mm)	Colonial Morphology	
	Type I	Type II
$\leq 2$	15	29
$> 2$	34	42
Total isolates	49	71

\* No significant difference between type of isolate and probing depth  
( $P > .05$ )

TABLE 4

Hemagglutination (HA) and Hemagglutination  
Inhibition (HI) by D-Galactose

<u>Microorganism</u>	<u>HA Titers*</u>	<u>HI Titer*</u>
ATCC	1:128	1:64
VPI 4355	1:128	1:64
Type I	1:128	1:32
Type I	1:256	1:32
Type I	1:128	1:32
Type I	1:128	1:32
Type II	1:128	1:32
Type II	1:128	1:32
Type II	1:128	1:32
Type II	1:128	1:32

\* Highest dilution displaying at 2+ hemagglutination

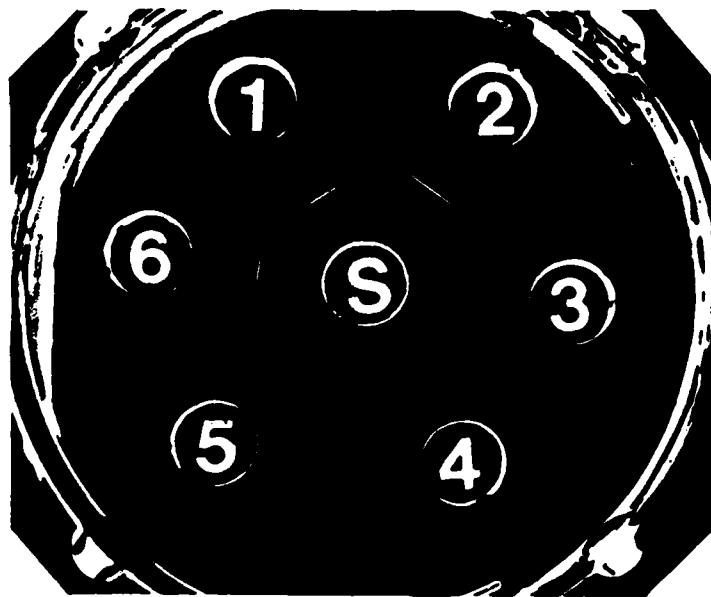


FIGURE 1. Double diffusion in agar using rabbit anti-F. nucleatum 10953 serum(s) with the SP of two typed stains (1,4); two Type I (2,5); and two Type II colonial morphology F. nucleatum (3,6).



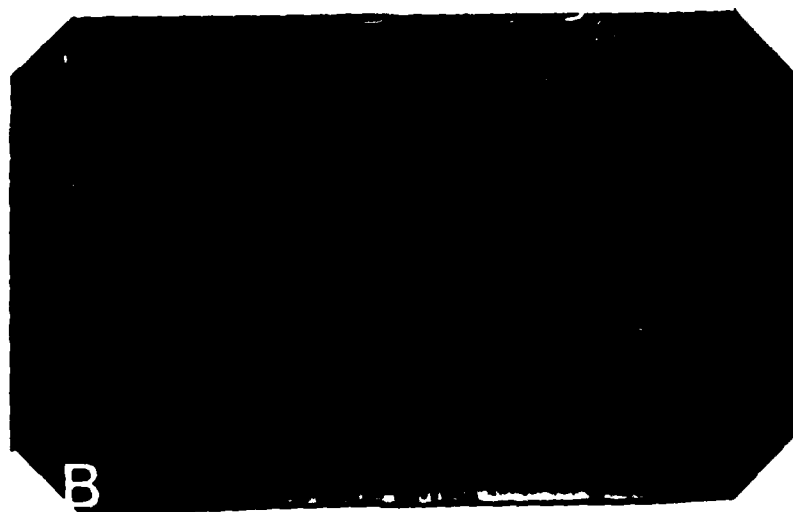


FIGURE 2. A: microtiter plate showing hemagglutinating activity of F. nucleatum isolates. B: reduction of hemagglutinating activity by exposure to 50 mM D-galactose.



FIGURE 3. PAGE slab gel showing protein patterns of low molecular weight proteins (<50,000) obtained utilizing two typed strains (4,11); five isolates with Type I colonial morphology (1,2,5,7,9) and five isolates with Type II colonial morphology (3,6,8,10,12).

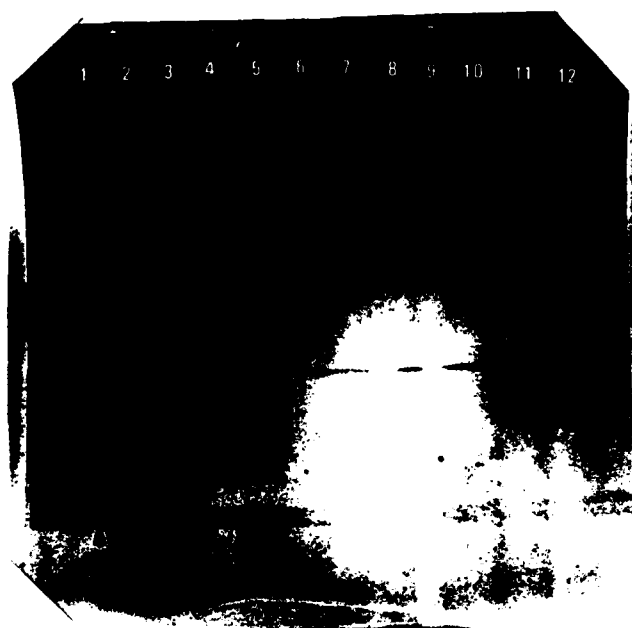


FIGURE 4. PAGE slab gel showing protein patterns of high molecular weight proteins (>50,000) obtained utilizing two typed strains (4,11); five isolates with Type I colonial morphology (1,2,5,7,9) and five isolates with Type II colonial morphology (3,6,8,10,12).

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